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Original citation:
James, Rob S. et al. "The effects of hibernation on the contractile and biochemical properties of skeletal muscles in the thirteen-lined ground squirrel, Ictidomys tridecemlineatus". Journal of Experimental Biology. 2013, 216. 2587-2594.
https://dx.doi.org/10.1242/jeb.080663

DOI 10.1242/jeb.080663
ISSN 0022-0949
ESSN 1477-9145

Publisher: Company of Biologists

The Journal of Experimental Biology 216, 2587-2594

doi:10.1242/jeb.080663

RESEARCH ARTICLE

The effects of hibernation on the contractile and biochemical properties of skeletal muscles in the thirteen-lined ground squirrel, *Ictidomys tridecemlineatus*

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SUMMARY

Hibernation is a crucial strategy of winter survival used by many mammals. During hibernation, thirteen-lined ground squirrels, *Ictidomys tridecemlineatus*, cycle through a series of torpor bouts, each lasting more than a week, during which the animals are largely immobile. Previous hibernation studies have demonstrated that such natural models of skeletal muscle disuse cause limited or no change in either skeletal muscle size or contractile performance. However, work loop analysis of skeletal muscle, which provides a realistic assessment of *in vivo* power output, has not previously been undertaken in mammals that undergo prolonged torpor during hibernation. In the present study, our aim was to assess the effects of 3 months of hibernation on contractile performance (using the work loop technique) and several biochemical properties that may affect performance. There was no significant difference in soleus muscle power output–cycle frequency curves between winter (torpid) and summer (active) animals. Total antioxidant capacity of gastrocnemius muscle was 156% higher in torpid than in summer animals, suggesting one potential mechanism for maintenance of acute muscle performance. Soleus muscle fatigue resistance was significantly lower in torpid than in summer animals. Gastrocnemius muscle glycogen content was unchanged. However, state 3 and state 4 mitochondrial respiration rates were significantly suppressed, by 59% and 44%, respectively, in mixed hindlimb skeletal muscle from torpid animals compared with summer controls. These findings in hindlimb skeletal muscles suggest that, although maximal contractile power output is maintained in torpor, there is both suppression of ATP production capacity and reduced fatigue resistance.

Key words: fatigue, power output, soleus, torpor, work loop.

Received 26 September 2012; Accepted 10 March 2013

INTRODUCTION

A wide variety of mammals hibernate during winter to reduce metabolic energy demands during seasonally low environmental temperatures and scarcity of food. Hibernation usually consists of a series of torpor bouts, during which core body temperature decreases towards ambient temperature, and metabolic rate may fall by >90% (Heldmaier et al., 2004; Staples and Brown, 2008; Storey et al., 2010). Mitochondrial respiration accounts for up to 90% of whole-animal oxygen consumption (Rolfe and Brown, 1997), and the decrease in whole-animal metabolism during torpor corresponds to reversible suppression of both liver and skeletal muscle mitochondrial respiration rates, by 70% and 30%, respectively, in thirteen-lined ground squirrels (Brown et al., 2012).

Skeletal muscle tissue plays an important role in enabling animals to undertake various fitness-related behaviours, including prey capture, predator avoidance and reproduction. Therefore, maintenance of skeletal muscle size and performance during periods of natural dormancy would be advantageous for fitness-related activities that occur upon arousal from hibernation. Clinical and experimental models of mammalian muscle disuse, such as limb immobilisation and hindlimb unloading, lead to rapid muscle atrophy and reduction in contractile performance (Musacchia et al., 1988; Powers et al., 2007; Clark, 2009). During torpor bouts, hibernators exhibit no discernible movement, yet previous studies on the effects of hibernation on skeletal muscle have demonstrated relatively low levels of muscle atrophy when compared with non-natural models of muscle disuse (Musacchia et al., 1988; Hudson and Franklin, 2002; Shavlakadze and Grounds, 2006). A range of mechanisms are thought to contribute to this resistance to skeletal muscle atrophy, including increased levels of antioxidants (Hudson and Franklin, 2002; Allan and Storey, 2012), reduced levels of myostatin (Braulke et al., 2010; Brooks et al., 2011; Nowell et al., 2011) and the regulation of transcription factors that transcribe genes associated with muscle performance (Tessier and Storey, 2010). Higher levels of antioxidants, such as glutathione and superoxide dismutase, are thought to counter the effects of reactive oxygen species (ROS), reducing cellular damage and potential muscle atrophy (Carey et al., 2003; Powers et al., 2007). However, in the thirteen-lined ground squirrel, there is evidence that many antioxidant enzymes are not upregulated during hibernation in brain, liver or heart tissue (Page et al., 2009).

Although skeletal muscle mass may decrease slightly during hibernation, previous studies have demonstrated limited or no change in contractile performance (Musacchia et al., 1988; Cotton and Harlow, 2010; James, 2010). James and co-workers demonstrated that daily torpor in the Djungarian hamster actually increased isometric force and work loop power output in soleus muscle but had no effect on either soleus or extensor digitorum longus (EDL)

work loop fatigue resistance (James et al., 2011). However, the Djungarian hamster spends only a few hours each day in torpor, while most hibernators undergo much longer torpor bouts that could cause more significant changes in contractile performance that might not have been detected *via* the use of isometric methods in all other previous studies on hibernators.

The primary aim of the present study was to determine the effects of hibernation, involving longer torpor bouts, on the contractile properties of hindlimb muscles in thirteen-lined ground squirrels. This is the first study to use the work loop technique to assess the effects of prolonged torpor bouts on contractile performance of mammalian skeletal muscle. The work loop technique assesses contractile performance of muscle while it undertakes cyclical length changes, providing a more realistic assessment of the likely *in vivo* power-generating capacity of muscle than isometric or force–velocity studies (Josephson, 1993; James et al., 1996). To evaluate potential underlying reasons for the observed muscle contractile properties and resistance to disuse atrophy, we also undertook measurements of antioxidant capacity, glycogen content and mitochondrial respiration rate in hindlimb muscle of the same animals. On the basis of previous studies that investigated contractile properties and size of skeletal muscle, we hypothesised that there would be limited differences in soleus muscle mass and acute contractile performance (peak force and power) between winter (torpid) and summer (active) animals. However, we also hypothesised that metabolic suppression (i.e. suppression of mitochondrial oxidative phosphorylation) during hibernation might lead to lower fatigue resistance in torpid compared with summer hindlimb muscle.

MATERIALS AND METHODS

Animals

All procedures were approved by the Animal Use Subcommittee at the University of Western Ontario (London, ON, Canada) where the experiments were performed. Adult thirteen-lined ground squirrels, *Ictidomys tridecemlineatus* (Mitchill 1821), were live-trapped (Tomahawk, Hazelhurst, WI, USA) from late May to early June in the wild (Carman, MB, Canada; 49°30′N, 98°01′W) and were transported by automobile to London, ON, Canada. Individuals were housed separately in shoebox-style cages (26.7×48.3×20.3 cm high) with dried corncob bedding, paper towels for nest building and a transparent red plastic tube (Bio-Serv, Frenchtown, NJ, USA) for enrichment. Animals arrived at the lab on 6 June 2011, where they were housed at 23±3°C with a photoperiod adjusted weekly to match that of Carman, MB, until October. Water and food (LabDiet 5P00, St Louis, MO, USA) were supplied *ad libitum*. Food was supplemented with 10–12 sunflower seeds three times weekly. Animals were weighed weekly at the time of cage changing and all animals displayed mass gain, from June through to August, characteristic of hibernators. In early July, 5–6 weeks following capture, all animals underwent minor surgery as described previously (Muleme et al., 2006) during which temperature-sensitive radio-telemetric transmitters were implanted intraperitoneally. These transmitters were used to measure body temperature to monitor torpor bouts.

Six animals (3 males, 3 females) were randomly selected and were killed in late July (summer animals) by barbituate overdose (Euthanyl, 270 mg ml^{-1}, 0.2 ml 100 g^{-1}). Euthanyl has no effect on mitochondrial metabolism (Takaki et al., 1997). Tissue samples were subsequently removed from these summer animals.

By early October, the remaining six animals (3 males, 3 females) had ceased to gain further mass, and many appeared to be torpid despite being held at 23°C. At this time, these animals were transferred to a controlled environment chamber and the temperature was reduced by 3°C each day until 5°C was reached. At this time, the photoperiod was reduced to 2 h light:22 h dark (Brown et al., 2012) (lights on at 09:00 h). These conditions induced torpor in all individuals within a week, at which time food was removed but water was supplied *ad libitum*. The six torpid animals were killed (see below), and skeletal tissue samples removed, ~3 months after their first torpor bouts were observed. In accordance with animal care protocols, torpid animals were euthanised by cervical dislocation to prevent arousal from torpor.

Dissection

Ground squirrel body mass was determined to the nearest 0.1 g using an electronic balance (model PJ300, Mettler, Zurich, Switzerland). Both hindlimbs were removed and were used for dissection. Gastrocnemius muscle samples were rapidly isolated from the right hindlimb, while kept at room temperature (19–22°C), then flash frozen in liquid nitrogen prior to storage in a −80°C freezer in preparation for subsequent analyses of glycogen content and antioxidant capacity. As much skeletal muscle as possible was removed from the hindlimb to produce a mixed hindlimb muscle sample for subsequent isolation of mitochondria. Soleus muscle was dissected from the left hindlimb in oxygenated (95% O_2: 5% CO_2) ice-cold Krebs–Henseleit solution (composition, in mmol l^{-1}: 118 NaCl, 4.75 KCl, 1.18 $MgSO_4$, 24.8 $NaHCO_3$, 1.18 KH_2PO_4, 10 glucose, 2.54 $CaCl_2$; pH 7.4).

Isometric studies

For each soleus muscle preparation, one tendon was clamped to a strain gauge (UF1, Pioden Controls, Canterbury, Kent, UK) whilst the other tendon was clamped to a motor arm (V201, Ling Dynamics Systems, Royston, Herts, UK) attached to an LVDT (linear variable displacement transformer, DFG 5.0, Solartron Metrology, Bognor Regis, West Sussex, UK). The muscle was then maintained at 36.5±0.5°C in circulating oxygenated (95% O_2: 5% CO_2) Krebs solution. The preparation was stimulated *via* parallel platinum electrodes while held at constant length to generate a series of twitches. Stimulus amplitude, pulse width (pulse duration) and muscle length were adjusted to determine the stimulation parameters and muscle length corresponding to maximal isometric twitch force. The muscle length that yielded maximal twitch force was measured to the nearest 0.1 mm using vernier calipers. An isometric tetanic force response was then elicited by subjecting the soleus muscle to a 350 ms train of stimulation. Time to half-peak tetanic force and time from last stimulus to half-tetanic force relaxation were measured. A rest period of 5 min was allowed between each tetanic response. Stimulation frequency was then altered to determine maximal tetanic force.

Work loop studies

The work loop technique was used to determine the power output of muscles during cyclical length changes (Josephson, 1985; James et al., 1995). Each muscle preparation was subjected to a set of four sinusoidal length changes symmetrical about the length that was optimal for maximal twitch force production. The muscle stimulation parameters found to yield maximal isometric force were used (stimulation frequency, amplitude and pulse width). Electrical stimulation and length changes were controlled *via* a data acquisition board (KUSB3116, Keithley Instruments, Cleveland, OH, USA) and a custom-designed program developed with TestPoint software (CEC Testpoint version 7, Measurement Computing, Norton, MA,

USA). For each work loop cycle, muscle force was plotted against muscle length to generate a work loop, the area of which equated to the net work produced by the muscle during the cycle of length change (Josephson, 1985). Instantaneous power output was calculated for every data point in each work loop (1000 data points per work loop), then these instantaneous power output values were averaged to generate a net work value for each work loop. The net work produced was multiplied by the frequency of length change cycles to calculate net power output (average power per cycle). A total strain of length change cycles of 0.10 was initially used in each experiment (i.e. ±5% of resting muscle length). During each work loop the muscle was subjected to phasic stimulation (active work loop cycle). Every 5 min the muscle was subjected to a further set of four work loop cycles with strain, stimulation duration and stimulation phase parameters being altered until maximum net work was achieved at each cycle frequency. The cycle frequency of length change was altered up and down within the range 1–5 Hz to generate power output–cycle frequency curves. Before the fatigue run, a set of control sinusoidal length change and stimulation parameters was imposed on the muscle every three to five sets of work loops to monitor variation in the muscle's ability to produce power/force. Any variation in power was found to be due to a matching change in the ability to produce force. Therefore, the power produced by each preparation, prior to the fatigue run, was corrected to the control run that yielded the highest power output, assuming that alterations in power generating ability were linear over time. On completion of the power output–cycle frequency curve, each muscle was subjected to a fatigue run consisting of 60 work loop cycles at a cycle frequency of 2 Hz, using the strain and stimulation parameters found to generate maximal power output. Within 5 min of completion of the fatigue run muscles on average had recovered to 61% of their pre-fatigue power output.

At the end of the muscle mechanics experiments, bones, tendons and connective tissue were removed and each soleus muscle was blotted on absorbent paper to remove excess Krebs solution. Wet muscle mass was determined to the nearest 0.1 mg using an electronic balance (model TR-204, Denver Instrument Company, Bohemia, NY, USA). Mean muscle cross-sectional area was calculated from muscle length and mass assuming a density of 1060 kg m^{-3} (Méndez and Keys, 1960). Maximum isometric muscle stress was then calculated as maximum tetanic force divided by mean cross-sectional area (kN m^{-2}). Normalised muscle power output was calculated as power output divided by wet muscle mass (W kg^{-1}).

Antioxidant capacity assay

An antioxidant assay kit was used (Cayman Chemical Company, Ann Arbor, MI, USA), which relies on the ability of antioxidants in the sample to inhibit oxidation of ABTS [2,2′-azino-di-(3-ethylbenzthiazoline sulphonate)]. A vial containing lyophilised powder of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was reconstituted in 1 ml of HPLC-grade water (final concentration, 1.5 mmol l^{-1}) and vortexed. The preparations for the standard curve combined increasing amounts of the reconstituted Trolox diluted in antioxidant assay buffer (5 mmol l^{-1} potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose). The following concentrations of Trolox were used to create the standard curve; 0, 0.045, 0.090, 0.135, 0.18, 0.225 and 0.330 mmol l^{-1}. The standard wells were prepared by adding 10 µl of a Trolox preparation, 10 µl of metmyoglobin (lyophilised powder reconstituted in antioxidant assay buffer) and 150 µl of chromogen (containing ABTS reconstituted in HPLC-grade water) per well. Samples of frozen gastrocnemius muscle were crushed into small pieces under liquid nitrogen, weighed, and then homogenised 1:4 w:v in ice-cold antioxidant assay buffer (containing protease inhibitor) as per the manufacturer's instructions. The samples were centrifuged at 10,000 r.p.m. (10 min, 4°C), and the supernatant collected and stored at −80°C until use. Sample wells were prepared by adding 10 µl of a 10-fold dilution of each muscle sample (diluted in antioxidant assay buffer), 10 µl of metmyoglobin, and 150 µl of chromogen. All samples and standards were analysed in duplicate. The reactions were initiated by addition of 40 µl of 441 µmol l^{-1} hydrogen peroxide followed by incubation on a shaker for 5 min at room temperature. The absorbance was read at 750 nm, using a plate reader, and quantified as Trolox equivalents (mmol l^{-1} mg^{-1} wet mass). Antioxidants measured by the assay include glutathione, ascorbate (vitamin C), vitamin E, bilirubin, BSA and uric acid.

Glycogen assay

A glycogen assay kit was used (Cayman Chemical Company). A vial containing lyophilised powder of glycogen was reconstituted in glycogen assay buffer (phosphate-buffered saline, pH 7.0) to a final concentration of 200 µg ml^{-1} and vortexed. The preparations for the standard curve combined increasing amounts of the reconstituted glycogen diluted in glycogen assay buffer. The following concentrations of glycogen were used to create the standard curve: 0, 2.5, 5, 10, 15, 20, 30 and 40 µg ml^{-1}. Samples of frozen gastrocnemius muscle were crushed into small pieces under liquid nitrogen, weighed and homogenised 1:4 w:v in ice-cold glycogen assay buffer (containing protease inhibitor) as per the manufacturer's instructions. The samples were centrifuged at 3000 r.p.m. (10 min, 4°C), and the supernatant collected and stored at −80°C until use. The standard and sample wells were prepared by adding 10 µl of standard or 10 µl of muscle sample per well, and 50 µl of reconstituted hydrolysis enzyme solution (lyophilised powder of amyloglucosidase reconstituted in a 50 mmol l^{-1} acetate glycogen hydrolysis buffer, pH 4.5). The sample background wells were prepared by adding 10 µl of the sample and 50 µl of the glycogen hydrolysis buffer (without amyloglucosidase). The plate was covered and incubated for 30 min at 37°C to allow for full glycogen hydrolysis. During the incubation, the developer was prepared by combining the following components: 0.5 ml fluorometric detector (lyophilised powder of 10-acetyl-3,7-dihydroxyphenoxazine, reconstituted in 100 µl DMSO and 400 µl assay buffer), 2.5 ml enzyme mixture (lyophilised enzyme mixture reconstituted in 2.5 ml assay buffer) and 5 ml assay buffer. Following a 30 min incubation, 150 µl of developer was added to all wells including the standard, sample and sample background wells. The plate was covered and incubated for 15 min at 37°C for maximum fluorescence development. The plate was read in a FluoStar plate reader (BMG Labtech, Allmendgruen, Ortenberg, Germany) using an excitation wavelength of 530–540 nm and an emission wavelength of 585–595 nm.

Mitochondrial respiration rates

Crude skeletal muscle mitochondria were isolated from mixed hindlimb skeletal muscle samples following a modified protocol (Bhattacharya et al., 1991) and purified using a previous method (Yoshida et al., 2007). Mixed muscle tissue was washed in ice-cold muscle homogenisation buffer (MHB, in mmol l^{-1}; 100 sucrose, 10 EDTA, 100 Tris-HCl, 46 KCl; pH 7.4 at 4°C). Fat, connective tissue, nerves and hair were removed. The remaining muscle tissue was decanted and suspended in nine volumes of MHB with protease (from *Bacillus licheniformis*, 5 mg g^{-1} wet muscle mass; Sigma, St Louis, MO, USA) and minced with fine scissors. After 5 min, muscle tissue

was homogenised with three passes of a loose-fitting Teflon pestle in a glass mortar. The homogenate was then incubated on ice for 5 min, followed by further homogenisation with three passes of a tight-fitting Teflon pestle in a glass mortar. The resulting homogenate was filtered through one layer of cheesecloth and centrifuged at 2000 ***g*** for 10 min at 4°C. The supernatant was filtered through four layers of cheesecloth and centrifuged at 10,000 ***g*** for 10 min at 4°C. The pellet was suspended in 5 ml MHB with 0.5% BSA and centrifuged again at 10,000 ***g*** for 10 min at 4°C. This pellet was then resuspended in 5 ml MHB, and this raw mitochondrial suspension was layered on top of 5 ml of 60% Percoll solution (made in MHB) and centrifuged at 21,000 ***g*** for 1 h at 4°C. Purified muscle mitochondria accumulated at the boundary between the MHB and 60% Percoll solution, and were removed and suspended in MHB. To remove residual Percoll, this suspension was centrifuged at 21,000 ***g*** for 10 min at 4°C. This washing step was repeated three times. The final pellet was used for all measurements of respiration rate.

Mitochondrial respiration rates were determined using a high-resolution respirometer (Oxygraph O2K, Oroboros, Innsbruck, Austria) calibrated with air-saturated buffer and oxygen-depleted buffer (obtained by addition of yeast suspension) using published oxygen solubilities (Forstner and Gnaiger, 1983), corrected for local atmospheric pressure. Unless otherwise noted, all compounds were dissolved in MiR05 assay buffer (in mmol l^{-1}: 110 sucrose, 0.5 EGTA, 3 $MgCl_2$, 60 potassium lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes; pH 7.1 at 30°C, 1% BSA) (Gnaiger and Kuznetsov, 2002). Respiration of skeletal muscle mitochondria was measured by adding ~0.03 mg protein to 2 ml of MiR05 assay buffer equilibrated to 37°C. Succinate (10 mmol l^{-1}; in the presence of rotenone, 2 μg ml^{-1}, dissolved in ethanol) was added to stimulate state 2 respiration, and state 3 respiration was achieved by adding ADP (0.2 mmol l^{-1}). Conditions approximating state 4 respiration were subsequently achieved by adding oligomycin (2 μg ml^{-1}, dissolved in ethanol).

Statistical analysis

Any dispersion measurements are given as s.e.m. In most cases, summer (active) and winter (torpid) results were compared using independent samples *t*-tests. Levene's test demonstrated that variances were equal in all cases. Comparison of power output–cycle frequency curve data between summer and torpid samples was performed using two-factor ANOVA with experimental treatment and cycle frequency as the factors. This approach allowed the interaction term to indicate whether torpor had affected the shape of the power output–cycle frequency curve. Hypothetically, if during torpor the contractile properties of a muscle became faster, due to a shift towards a faster muscle fibre type, there might be a rightward and upward shift in the peak of that muscle's power output–cycle frequency curve.

Two-factor ANOVA was also used to analyse the effects of torpor on fatigue resistance, with hibernation and loop number (essentially, time elapsed during the fatigue run) used as the two factors. The interaction term in this analysis should indicate whether the shape in the curve is different, hence indicating a difference in the pattern of fatigue between torpid and summer animals. Independent samples *t*-tests were used to determine whether power output was significantly different between torpid and summer animals at each time point during the fatigue run.

The truncated product method (Zaykin et al., 2002) was used to combine all the *P*-values in this study to determine whether there was a bias from multiple hypothesis testing. The truncated product method *P*-value was <0.001, indicating that the results were not biased by multiple comparisons.

Table 1. Effects of hibernation on soleus muscle and body size in thirteen-lined ground squirrels

	Torpid	Summer	*P*
Muscle length (mm)	23.5±1.5	25.9±1.2	0.25
Muscle mass (mg)	73.9±4.6	86.2±5.4	0.13
Muscle mass/body mass (%)	0.0517±0.0053	0.0537±0.0042	0.77
Body mass (g)	145±6	165±17	0.35

Data represent means ± s.e.m. *P*-values are given for independent samples *t*-tests. *N*=5 for torpid and *N*=6 for summer animals.

RESULTS

There were no significant differences in soleus muscle length, soleus muscle mass or body mass between summer and torpid thirteen-lined ground squirrels (Table 1). Soleus muscle mass, expressed as a percentage of body mass, also did not differ significantly between torpid and summer animals (Table 1).

Isometric properties

There were no significant differences in the isometric properties of soleus muscle between summer and torpid thirteen-lined ground squirrels (Table 2). However, there was a tendency for summer animals to have faster soleus muscle tetanus relaxation times and higher maximal tetanic stress ($0.05<P<0.10$ in each case).

Work loop performance

Soleus muscle work loop power output (standardised to muscle mass) tended to be higher in torpid than in summer thirteen-lined ground squirrels (Fig. 1; ANOVA main effect, $F=3.49$, $P=0.068$). There was no significant change in the shape of the power output–cycle frequency curve (ANOVA interaction term, $F=0.16$, $P=0.957$). Therefore, the power output–cycle frequency curves looked very similar for torpid and summer animals.

Performance during a fatigue test was initially identical between summer and torpid animals, but then soleus muscle power output declined at a more rapid rate in torpid than in summer animals (Fig. 2). Therefore, summer animals produced higher power output than torpid animals during the fatigue test (ANOVA main effect, $F=68.0$, $P<0.001$). From loop 18 onwards, soleus muscle power output was a significantly (*t*-test, $P<0.05$ in each case) higher percentage of maximal power output in summer than in torpid animals (Fig. 2). There was also a significant difference in the shape of the fatigue curves (ANOVA interaction term, $F=4.86$, $P<0.001$), indicative of the more rapid fatigue of soleus muscle from torpid animals when compared with summer animals. In both summer and torpid animals, the key cause of fatigue was a reduction in force causing a drop in force production during the work loop (Fig. 3).

Table 2. Effects of hibernation on soleus muscle tetanus kinetics and maximal isometric stress in thirteen-lined ground squirrels

	Torpid	Summer	*P*
Time to half-peak tetanus (ms)	56.6±5.8	50.9±2.9	0.37
Time from last stimulus to half-tetanus relaxation (ms)	82.6±5.1	67.7±4.3	0.05
Maximum twitch stress (kN m^{-2})	65.3±6.5	56.9±10.5	0.54
Maximum tetanic stress (kN m^{-2})	280±5	366±69	0.08

Data represent means ± s.e.m. *P*-values are given for independent samples *t*-tests. *N*=5 for torpid and *N*=6 for summer animals.

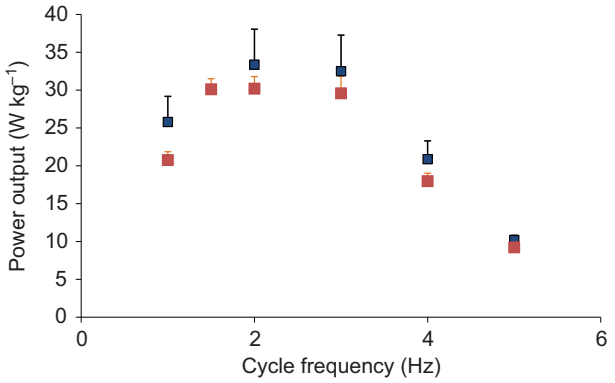

Fig. 1. Soleus work loop power output (standardised to muscle mass) was not significantly different between summer (red; *N*=6) and torpid (blue; *N*=5) thirteen-lined ground squirrels (*P*=0.068). Data represent means + s.e.m.

Antioxidants

The total antioxidant capacity of the thirteen-lined ground squirrel gastrocnemius muscle was measured as Trolox equivalents and increased significantly such that it was 156% higher in torpid animals than in summer animals (Fig. 4; t=8.25, P<0.001).

Glycogen

Gastrocnemius muscle glycogen content was not significantly different between summer (15.2±0.7 µmol glucosyl units gram^{-1} wet mass) and torpid (14.8±0.2 µmol glucosyl units gram^{-1} wet mass) thirteen-lined ground squirrels (t=0.50, P=0.64).

Mitochondrial respiration rates

Mixed hindlimb muscle state 3 mitochondrial respiration rate was 145% higher in summer than in torpid thirteen-lined ground squirrels (t=3.66, P=0.005; Fig. 5). Mixed hindlimb muscle state 4 mitochondrial respiration rate was 79% higher in summer than in torpid thirteen-lined ground squirrels (t=3.21, P=0.012; Fig. 5).

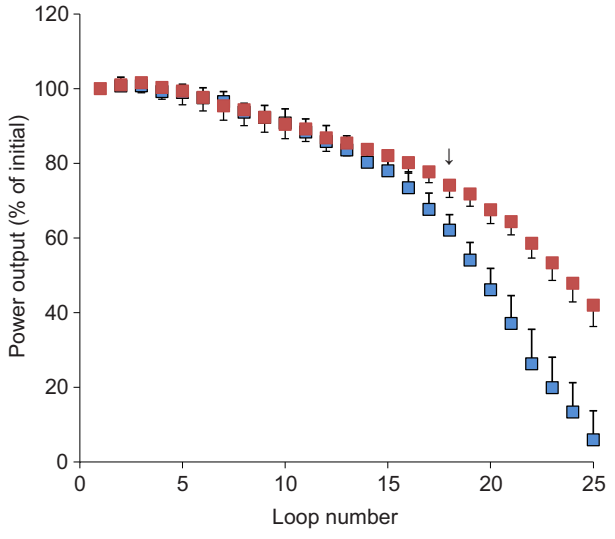

Fig. 2. Soleus muscle fatigue resistance, during a series of work loops, was lower in torpid (blue; *N*=5) than in summer (red; *N*=6) thirteen-lined ground squirrels (ANOVA main effect, *F*=68.0, *P*<0.001). Beyond loop 18, indicated by an arrow, power output was significantly lower in the torpid group. For each muscle, power output was standardised as a percentage of the maximal power output produced during any work loop in that muscle's fatigue run. Data represent means ± s.e.m.

DISCUSSION

Skeletal muscle size

Consistent with our hypothesis, we found that soleus muscle size was not significantly different between torpid and summer thirteen-lined ground squirrels. Our measurements of soleus muscle mass and subsequent expression of muscle mass as a percentage of body mass suggest that atrophy of this muscle, of relatively slow fibre type, is limited during hibernation in this species as there was little indication of atrophy in these squirrels following hibernation for ~3 months. Furthermore, even if atrophy does occur, the maintenance of soleus muscle size with respect to body size is in keeping with the idea that maintenance of muscle size is important to preserve acute contractile and locomotor performance. Nowell and co-workers indicated that there was no significant change in soleus and diaphragm muscle mass during hibernation in golden-mantled ground squirrels (Nowell et al., 2011). In contrast, there was significant (~25%) atrophy during hibernation in muscles of faster fibre type (plantaris and gastrocnemius), but this atrophy occurred early in the hibernation season, with limited atrophy during the remainder of hibernation (Nowell et al., 2011). Nowell and co-workers also demonstrated a significant reduction in myostatin mRNA expression in soleus and diaphragm muscles, but not in limb muscles of faster fibre type, which may be a key mechanism for reducing atrophy during hibernation (Nowell et al., 2011).

In thirteen-lined ground squirrels, myostatin protein levels in mixed hindlimb muscle were generally constant throughout torpor as compared with controls, but rose significantly during arousal (Brooks et al., 2011). Also, during arousal from hibernation, many hibernators undergo shivering thermogenesis, during which there is vigorous activity of muscles, which may also play a role in maintaining muscle mass (Lee et al., 2010). Regardless, in various studies of mammalian hibernation, including other studies of ground squirrels, there does not seem to be any consensus as to the degree of muscle atrophy and, more importantly, the direct mechanisms (e.g. muscle fibre type shifts) responsible for resistance to atrophy (Musacchia et al., 1988; Rourke et al., 2004; Lohuis et al., 2007; Cotton and Harlow, 2010; Gao et al., 2012). Nonetheless, the extent of muscle atrophy in mammalian hibernation is clearly lower than would be predicted from non-natural models of muscle disuse.

Contractile properties of skeletal muscle

As hypothesised, we found that acute contractile performance in soleus muscle was not significantly different between torpid and summer thirteen-lined ground squirrels. Our results indicate that soleus muscle in this species is maintained, both structurally and mechanically, during winter to be ready for emergence from hibernation. There was a tendency for peak soleus muscle power output (standardised to muscle mass) to be 10% higher in torpor than in summer; however, consideration of the overall shape of the power output–cycle frequency curve suggests very limited differences between the torpid and summer animals. The only previous study to use the work loop technique to analyse contractile properties in mammalian torpor found that soleus muscle power output (standardised to muscle mass) was significantly (27%) higher in torpid than in summer active Djungarian hamsters (James et al., 2011). However, that study found that there were no significant differences between torpid and summer animals in EDL muscle power output (James et al., 2011). In the present study, soleus contraction kinetics and the power output–cycle frequency curves

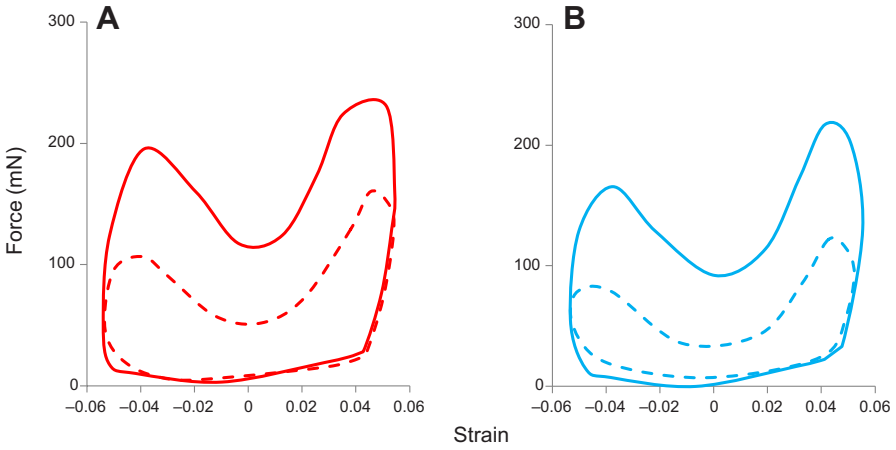

Fig. 3. Typical work loop shapes during the soleus muscle fatigue run for (A) summer (red) and (B) torpid (blue) groups. The solid lines represent the first work loop and the dashed lines represent the 20th work loop in a typical fatigue run.

showed very limited differences between summer and torpid animals.

In the present study, there is little evidence for differences in either 'speed'-related contractile properties or, consequently, fibre type (fast *versus* slow) of soleus muscle between summer and torpid thirteen-lined ground squirrels. Previous studies analysing the expression of myosin heavy chain (MHC) isoforms have shown limited or no change in fibre type during hibernation in relatively slow muscles (diaphragm and soleus) in golden-mantled ground squirrels (Rourke et al., 2004; Nowell et al., 2011). However, those studies demonstrated significant seasonal changes in myosin fibre type, early in hibernation, in relatively fast muscles (plantaris and gastrocnemius) in golden-mantled ground squirrels, with an apparent fast to slow transition in the expression of fast MHC isoforms (transition from IIB to IIx), but maintenance of the percentage of muscle composed of the type 1 MHC isoform (Rourke et al., 2004; Nowell et al., 2011). In contrast, Gao and co-workers demonstrated that another relatively fast hindlimb muscle, EDL, did not undergo shifts in muscle fibre type distribution during hibernation in dauria ground squirrels (Gao et al., 2012). When the hibernation literature is considered as a whole, the patterns of maintenance of fibre type or shift towards more oxidative fibre type during torpor/inactivity suggests a mechanism of atrophy resistance that is not shared with non-hibernating species. In contrast, muscle disuse in non-hibernating species tends to lead to a shift towards less aerobic (faster) fibre types, partly *via* greater atrophy of slow muscle fibres (Musacchia et al., 1988; Baldwin and Haddad, 2001; Thompson, 2002).

In the present study, we did demonstrate a significantly higher soleus muscle fatigue resistance in summer compared with torpid thirteen-lined ground squirrels. The only previous study to use work loops to test fatigue resistance in mammalian muscle subjected to torpor bouts found no significant differences in fatigue resistance between torpid and summer hamsters, in EDL or soleus muscles (James et al., 2011). Hibernation has previously been found to reduce fatigue resistance during isometric tetanus studies in the relatively fast twitch tibialis anterior muscle of wild black bears (Lohuis et al., 2007). However, with relatively few studies analysing fatigue resistance, it is difficult to speculate as to whether the differences found in our studies using ground squirrels and hamsters might relate to different mechanisms associated with differences in the length of torpor bouts.

Antioxidants

The present study indicated that total antioxidant capacity in the relatively fast twitch gastrocnemius muscle was significantly (156%) higher in torpid than in summer thirteen-lined ground squirrels. A

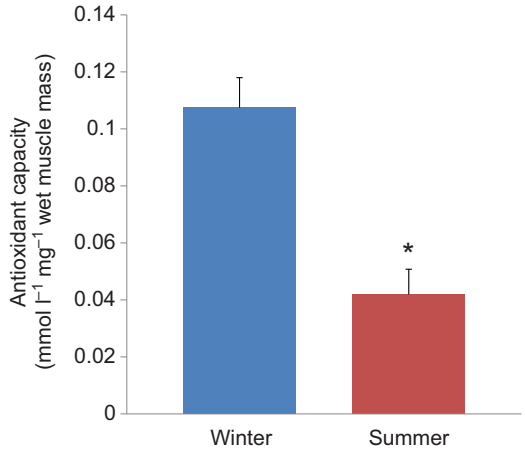

Fig. 4. Gastrocnemius muscle antioxidant capacity, measured as Trolox equivalents (mmol l^{-1} mg^{-1} wet mass), was significantly higher in torpid (winter; *N*=3) than in summer (*N*=3) thirteen-lined ground squirrels (**P*<0.001). Data represent means + s.e.m.

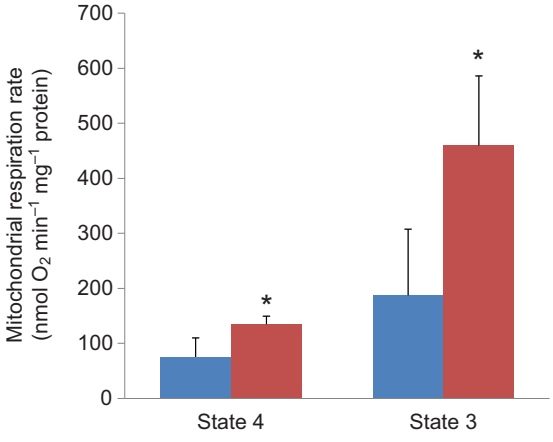

Fig. 5. Mixed hindlimb muscle state 3 and state 4 mitochondrial respiration rates were significantly lower in torpid (blue) than in summer (red) thirteen-lined ground squirrels (**P*=0.005 and *P*=0.012, respectively). Data represent means + s.e.m.; state 4, *N*=6 for torpid, *N*=4 for summer; state 3, *N*=6 for torpid, *N*=5 for summer.

higher production of ROS during muscle disuse can lead to increased proteolysis, muscle atrophy and decreases in contractile performance (Powers et al., 2007). Therefore, a higher antioxidant capacity during hibernation may protect the intracellular environment by scavenging ROS and related molecules. Enhanced antioxidant capacity during hibernation may have two general functions (Storey et al., 2010). Firstly, enhanced antioxidant capacity contributes to cytoprotection during torpor to provide greater protection of macromolecules at a time when the ability to replace oxidatively damaged molecules is reduced as a result of strong metabolic rate depression, which includes suppressed rates of transcription, translation and other forms of biosynthesis. Secondly, a well-developed antioxidant capacity is crucial to deal with a rapid increase in ROS production caused when the rate of oxygen consumption increases by as much as 30-fold when animals rewarm during interbout arousal (Boyer and Barnes, 1999). The findings of the current study are interesting as previous work on thirteen-lined ground squirrels found that antioxidant enzyme activities, including superoxide dismutase, glutathione peroxidase and glutathione reductase, were not upregulated during hibernation in brain, heart and liver tissues when compared with summer levels (Page et al., 2009), although antioxidant enzyme activities do increase in liver tissue during hibernation in other ground squirrel species (Buzadzić et al., 1992). However, there is evidence that levels of antioxidant metabolites are elevated; for example, ascorbate levels rise 3- to 5-fold in plasma during torpor and ascorbate is redistributed from plasma to liver and spleen tissues, at the same time as oxygen consumption peaks, during arousal from torpor in Arctic ground squirrels (Tøien et al., 2001; Drew et al., 2002). Elevated levels of oxidative stress markers were reported in brown adipose tissue (but not liver) of Arctic ground squirrels during arousal compared with torpor (Orr et al., 2009) indicating that this thermogenic organ experiences ROS challenge during arousal. As skeletal muscle is the other thermogenic tissue that powers arousal, enhanced antioxidant capacity in the muscle of torpid animals would be a valuable preparation for muscle to deal with oxidative stress during arousal. Therefore, regulation of antioxidant activity during hibernation can differ between tissues in the same species. Further work needs to be undertaken to determine whether increased antioxidant capacity is a general response to hibernation that occurs in other skeletal muscles, including those of slower fibre type, of this species. In addition to increased antioxidant activity, there is evidence that mitochondrial ROS production may be reduced during torpor in mixed hindlimb muscle, especially *via* passive temperature effects, and that this may further reduce oxidative damage to muscle in hibernating animals (Brown et al., 2012).

Glycogen

As a general fuel for intermediary metabolism, depleted glycogen stores might exist in torpid animals resulting in muscles that fatigue more quickly than those of summer animals. However, in the present study glycogen levels were not significantly different, in gastrocnemius muscle, between summer and torpid thirteen-lined ground squirrels. During torpor, mammals primarily mobilise fatty acids for fuel (Heldmaier et al., 1999; Buck and Barnes, 2000; Carey et al., 2003). Therefore, glycogen stores may be kept constant throughout hibernation or may be reserved for more metabolically demanding time points such as during interbout arousal with its shivering thermogenesis. Future assessment of other skeletal muscles, including the more aerobic soleus muscle, would be useful to determine whether glycogen content is generally maintained in all muscles during hibernation. Maintaining basal levels of glycogen during torpor may be gated by enzymes which are actively regulated during hibernation. Evaluating proteins involved in glycogen regulation (such as glycogen phosphorylase or glycogen synthase), over a time course of torpor–arousal, would further elucidate how/whether glycogen stores are regulated. Glycogen phosphorylase activity in liver was shown to decrease during torpor in golden-mantled ground squirrels, whereas levels in mixed hindlimb skeletal muscle remain the same, consistent with the idea that carbohydrate reserves will be conserved during torpor in skeletal muscle (Brooks and Storey, 1992).

Mitochondrial respiration rates

In the present study, mixed hindlimb muscle mitochondrial respiration rates were significantly higher in summer than in torpid animals for state 3 and state 4 respiration (145% and 79% higher, respectively). These findings reinforce the idea that skeletal muscle oxidative capacity is decreased during winter torpor, with rapid reversal on arousal from torpor (Brown et al., 2012). These findings may also at least partly explain the lower fatigue resistance measured in soleus from torpid animals when compared with summer animals in the present study. Future work should measure both mitochondrial respiration rate and fatigue resistance of the same skeletal muscle during torpor as well as periods of interbout euthermia (when mitochondrial oxidative capacity is briefly restored) in hibernating animals to further investigate whether there is a direct effect of reduced mitochondrial respiration rate on fatigue resistance.

Conclusions

The findings from the present study indicate that thirteen-lined ground squirrels maintain skeletal muscle mass and acute contractile performance of soleus muscle during hibernation. These findings are consistent with the theory that, in natural models of muscle disuse, animals will attempt to maintain skeletal muscle performance ready for arousal (James, 2010). The higher levels of antioxidants in gastrocnemius muscle during torpor in thirteen-lined ground squirrels are consistent with this being a key mechanism for reducing muscle atrophy during disuse; however, we do not yet know whether this is a general response in all hindlimb skeletal muscle, including the more aerobic soleus muscle, in this species. Soleus muscle fatigue resistance was significantly lower in torpid than in summer thirteen-lined ground squirrels. There were no significant differences in glycogen stores between summer and torpid gastrocnemius muscle; however, we do not yet know whether this will be a general finding in all hindlimb skeletal muscle, including the more aerobic soleus muscle, in this species. Metabolic suppression during deep torpor, as evidenced by lower skeletal muscle mitochondrial respiration rates, may well limit the skeletal muscle capacity to undertake prolonged exercise, therefore reducing fatigue resistance. Further work is required to elucidate the detailed effects of hibernation on the contractile properties of relatively fast muscles.

ACKNOWLEDGEMENTS

Thanks to Jan Storey for comments on an earlier version of the manuscript. Dillon Chung and Vivian Jia provided invaluable animal husbandry support. We thank Alvin Iverson and the staff of the Carman and Area Research Facility (Manitoba) for their help in trapping animals. Thanks to two anonymous referees for their constructive comments that helped to improve the manuscript.

AUTHOR CONTRIBUTIONS

This study was conceived and designed by R.S.J., J.F.S. and K.B.S. All authors were involved in experimental measurements and interpretation of their findings. R.S.J., J.F.S., J.C.L.B. and K.B.S. were involved in drafting and revising this article.

COMPETING INTERESTS

No competing interests declared.

FUNDING

This research was supported by: a Coventry University Applied Research Fellowship that funded R.J. for travel to the University of Western Ontario and accommodation in January 2011; a grant from the University of Western Ontario Visiting Scholars Program that funded R.J. for travel to the University of Western Ontario, accommodation and a contribution towards research expenses in summer 2011; the Natural Sciences and Engineering Research Council (Canada) *via* Discovery, Research Tools and Infrastructure grants (J.F.S., K.B.S.), and Postgraduate Scholarships (J.C.L.B., S.N.T.).

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